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Tolerance of a field grown soybean cultivar to elevated ozone level is concurrent with higher leaflet ascorbic acid level, higher ascorbate-dehydroascorbate redox status, and long term photosynthetic productivity

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Abstract

We examined the characteristics of ascorbic acid (ASC) level, dehydroascorbate (DHA) level, and the ASC–DHA redox status in the leaflets of two soybean cultivars grown in a field environment and exposed to elevated ozone (O_3) levels. These two cultivars, one that preliminary evidence indicated to be O_3-tolerant (cv Essex), and one that was indicated to be O_3-sensitive (cv Forrest), were grown in open-top chambers during the summer of 1997. The plants were exposed daily to a controlled, moderately high O_3 level ($\approx$58 nl l^{-1} air) in the light, beginning at the seedling stage and continuing to bean maturity. Concurrently, control plants were exposed to carbon-filtered, ambient air containing a relatively low O_3 level ($\approx$24 nl l^{-1} air) during the same period. Elevated O_3 did not affect biomass per plant, mature leaf area accretion, or bean yield per plant of cv Essex. In contrast, elevated O_3 level decreased the biomass and bean yield per plant of cv Forrest by approximately 20%. Daily leaflet photosynthesis rate and stomatal conductance per unit area did not decrease in either cultivar as a result of prolonged O_3 exposure. A 10% lower mature leaflet area in O_3-treated cv Forrest plants contributed to an ultimate limitation in long-term photosynthetic productivity (vegetative and bean yield). Possible factors causing cv Essex to be more O_3 tolerant than cv Forrest were: 1) mature leaflets of control and O_3-treated cv Essex plants consistently maintained a higher daily ASC level than leaflets of cv Forrest plants, and 2) mature leaflets of cv Essex plants maintained a higher daily ASC–DHA redox status than leaflets of cv Forrest plants.

Abbreviations: ASC – L-ascorbic acid; ASC peroxidase – ascorbate peroxidase; DHA – dehydroascorbate; ASC + DHA – total ascorbic acid; ASC-GSH cycle – ascorbate-glutathione cycle – a system of enzymes for the recycling of DHA to ASC, including GSSG reductase, MDHA reductase and DHA reductase; Ce – gaseous CO_2 concentration in the measuring cuvette during photosynthesis measurement; Chl – chlorophyll; Ci – gaseous CO_2 concentration in the leaf intercellular spaces during photosynthesis measurement; G_s – stomatal conductance with units as mmol H_2O released m^{-2} s^{-1}; GSSG – oxidized glutathione; GSH – reduced glutathione; MDHA – monodehydroascorbate; PE – post-emergence; Pn – net CO_2 photoassimilation in leaves; PPF – photosynthetic photon flux with units of μmol photons m^{-2} s^{-1}

Introduction

Elevated ozone (O_3) levels in the United States and Canada cause a loss to agricultural productivity of as much as three billion dollars yearly (Heagle 1989; Heagle et al. 1998). Moderately high levels of O_3 (60–100 nl l^{-1} air) in the troposphere of soybean growing areas, e.g. mid-Atlantic states of the US, may cause

as much as a 40% loss of yield (Mulchi et al. 1988; Heagle 1989; Heagle et al. 1998). The bean yield of even the most O_3-tolerant soybean cultivars decreases when the O_3 concentrations are consistently in the range of 60–100 nl l^{-1} air during the entire growing season (Mulchi et al. 1988; Heagle 1989; Heagle et al. 1998). Thus, it is important to understand how soybean plants in field environments are able to tolerate O_3-mediated oxidative stress, especially during the periods which are vital to successful production of the beans.

Exposure of plants to elevated concentrations of O_3 is accompanied by an increase in hydroxyl radicals, superoxide radicals and H_2O_2, first in the apoplast and then in the symplast of leaf cells. Hydrogen peroxide, generated from O_3 decomposition in the apoplast and symplast, is removed by ASC peroxidases as well as by other general peroxidases which also may use ASC as the reductant. The resulting DHA and MDHA is recycled to ASC via the ASC–GSH cycle enzymes (Castillo and Greppin 1988; Polle et al. 1990; Luwe et al. 1993; Polle et al. 1995; Luwe and Heber 1995; Rao et al. 1995; Luwe 1996; Ranieri et al. 1996; Noctor and Foyer 1998; Lyons et al. 1999).

An important factor in the maintenance of ASC level is the photosynthetic production of glucose which is the precursor of galactose, the apparent direct precursor of ASC (Loewus 1988; Smirnoff 1996; Wheeler et al. 1998; Conklin et al. 1999; Davey et al. 1999; Loewus 1999; Pallenca and Smirnoff 1999). Ozone may inhibit CO_2 photoassimilation in leaves (Heath 1994, 1996) and/or reduce leaf area resulting in long term reductions in photosynthetic productivity; this may also lead to reduction in total ascorbic acid levels (ASC + DHA).

There is evidence that if leaves have higher ASC levels, the plants are more tolerant to elevated O_3. For example, when the soybean cultivars Hood and Hark were exposed to very high O_3 levels (250 nl l^{-1} air for 3 hours), ASC level in the leaflets of the O_3-tolerant cultivar (cv Hood) was as much as 20% higher than in the leaflets of the O_3-sensitive soybean cultivar (cv Hark) (Lee et al. 1984). More recently, Conklin et al. (1996) have developed a mutant of *Arabidopsis thalliana* that possesses only approximately 30% of the normal foliar level of ASC of the wild type. During severe acute O_3 treatment (400 nl l^{-1} air for 4 h), the low ASC mutant sustained at least twice the leaf damage as did the wild type. Interestingly, O_3 tolerance (prevention of leaf cell plasmalemma and enzyme damage) in both the ASC deficient mutant and the wild type *Arabidopsis* was increased when Conklin et al. (1996) supplied additional ASC to the pots in which the plants were growing.

There is also the possibility that plants that are able to maintain a higher foliar ASC to DHA redox status are more O_3-tolerant. For example, in our 1995 field study, two soybean cultivars were exposed during the growing season to elevated O_3 levels ($\approx$60 nl l^{-1} air). During elevated O_3 exposure, mature leaflets of an apparent O_3-tolerant soybean cultivar, cv Essex, maintained a higher ASC–DHA redox status in the midafternoon than did leaflets of the soybean cultivar cv Forrest which was thought to be more O_3 sensitive (Robinson et al. 1998; Robinson and Britz 2001). This was found to be the case in the vegetative, flowering and podding stages. However, in that study, a thorough examination of the influence of O_3 exposure on growth and yield parameters was not concurrently conducted (Robinson et al. 1998; Robinson and Britz 2001). Thus, there was neither vegetative shoot mass yield nor bean yield data in the 1995 experiments with which to compare cv Essex with cv Forrest for long term ozone tolerance.

A major purpose of this study was to expose field grown cv Essex and cv Forrest, for the entire growth and reproductive period, to moderately high O_3 levels, and to document the influence of elevated O_3 level on: 1) foliar photosynthesis rate, 2) stomatal conductance, 3) vegetative and reproductive yield, 4) foliar ASC and DHA levels, and 5) foliar ASC–DHA redox status. The results strongly suggest that the O_3-tolerant cultivar (cv Essex) had a higher daily foliar ASC level and ASC–DHA redox status than did the O_3-sensitive cultivar (cv Forrest) during the vegetative and reproductive periods. The implications of these observations are discussed.

Materials and methods

Plant growth

Seeds of cv Essex and cv Forrest were planted in fertilized soil held in compartmentalized plastic flats. The emerged seedlings were grown in the greenhouse to 10 d PE, at which time they were transported to the field plots for transplanting. In each of four replicate chambers fitted with carbon filters to remove some of the O_3 from the air flow (CF chambers), 50 seedlings of each cultivar were transplanted. Concurrently, in each of four replicate chambers equipped for O_3 injection into

the air flow (NF+O_3 chambers), 50 seedlings of each cultivar were transplanted. The floor of each chamber was divided into 4 quadrants with 25 cv Essex plants or 25 cv Forrest plants inserted in rows in each alternate quadrant. The plants remained in the chambers from the seedling stage, beginning in early June, to the bean harvest stage ending in October 1997.

Methods and equipment employed in these studies for propagation and chronic O_3 exposure of plants in open-top field chambers have been described in detail in other reports (Heagle et al. 1979; Mulchi et al. 1988). In CF chambers, ambient air was continuously fan-driven through a carbon filter with the air flow directed into a connecting duct and into the perforated lower duct panel, where the flow was driven into the lower part of the chamber, and ultimately, exited the open-top portion of the upper chamber. Carbon-filtered air flow, which removed some O_3, was continued 24 h daily.

In NF+O_3 chambers, during a daily period from 0900 to 1600 hrs, fan-driven ambient air was continuously mixed with metered amounts of O_3 at approximately 20 nl l^{-1} air above the level in the ambient air to achieve an average O_3 concentration approaching 58–60 nl l^{-1} air. The mixture was directed into the chambers as described for CF chambers.

Ozone was generated by passing a stream of pure O_2 through a high voltage discharge generator (Griffin[1] Ozone Generator, Model GTC1A). Ozone levels in ambient air were monitored with a Bendix[1] O_3 analyzer while within open-top chambers, O_3 levels in the air were monitored with a Dasibi[1] Model 1003 PC monitor. Ozone levels in the chambers were continuously analyzed; air samples in the chambers were extracted via tubing installed in the chambers and samples were channeled into the monitor (Mulchi et al. 1988).

Beginning at 10 d PE and continuing through bean harvest at 144 d PE, cultivars in CF chambers were supplied for 24 h daily, 7 d weekly with carbon-filtered air. The average O_3 level in the air during the period 0900 to 1600 hrs was approximately 24 nl l^{-1} (Figure 1). During the same period, plants in NF+O_3 chambers were supplied for 7 h daily (0900 to 1600 hrs) and 7 d weekly with an average elevated O_3 level of 58 nl l^{-1} air (Figure 1). During the other 17 hours of the day, plants in NF+O_3 chambers received non-filtered ambient air.

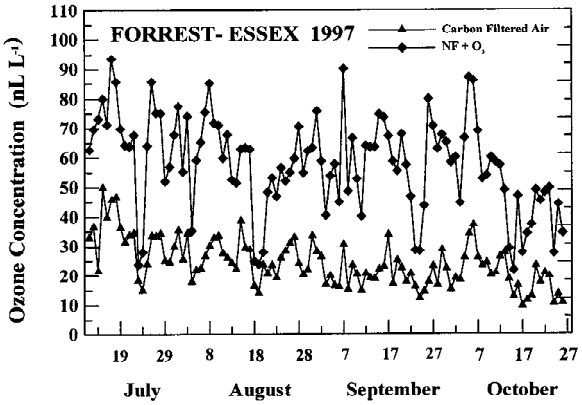

Figure 1. Average 7-h O_3 level profiles in open top chambers from early July (seedling stage) through the harvest in late October 1997 in open-top chambers. Ozone treatment was repeated 7 days per week during the entire growing and maturation season. Daily 7-h averages (n = 4 chambers) for CF chambers was ≈24 nl O_3 l^{-1} air (in the light). In nonfiltered (NF+O_3) open top chamber, additional ozone was injected into the nonfiltered air, so that the average O_3 concentration (n = 4 chambers) was maintained at ≈58 nl O_3 l^{-1} air (in the light).

Yield analyses

This study was conducted to determine the effect of elevated O_3 upon vegetative growth and bean yield of cv Essex and cv Forrest. The elevated level of O_3 (≈58 nl l^{-1} air) was preselected so there would be no visible damage to the measured leaves, but at the same time the elevated O_3 levels would be environmentally relevant (Figure 1). Tolerance or susceptibility to O_3 was judged, in part, by the influence of O_3 on shoot biomass per plant and mature leaf area (3 leaflets per leaf) of plants at 65 d PE and 40 d of elevated O_3 treatment. At 65 d PE, the above ground mass of each of 9 plants of each cultivar (in each CF or NF+O_3 chamber) was selected at random and harvested. From each cultivar (9 plants in each of 4 chambers, n = 36 plants), 1 mature leaf set of 3 leaflets (≈225–250 cm^2) was selected at random from the upper part of each of the plants, the total leaf area was determined employing a LI-COR 3100 area meter, the 3 leaflets were placed in a bag, oven dried, and subsequently weighed. Concurrently, the rest of each above ground plant mass from which the leaf was removed was placed in individual bags, oven dried, and subsequently the shoot dry mass was weighed. For each cultivar, shoot dry mass in each treatment included the weight of the leaf

[1] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products or vendors that may be suitable.

Table 1. Influence of elevated O_3 on cv Essex and cv Forrest growth parameters: (A) shoot biomass per plant (n = 36); (B) mature leaf area (3 leaflets per leaf) (n = 36) at 65 d PE and 40 d O_3 treatment; and (C) bean yield per plant (n = 40) at 144 d PE and 107 d O_3 treatment

Cultivar	cv Essex	cv Forrest
(A) Shoot biomass per plant[a,b]		
Treatment	gm plant^{-1}	
CF	16.6 ± 0.7	19.2 ± 1.0**d**
NF+O_3	17.4 ± 0.8	14.7 ± 0.6**fd**
(B) Area per mature leaf[a,b]		
Treatment	cm^2	
CF	236.3 ± 5.0	224.1 ± 5.9**d**
NF+O_3	232.2 ± 3.7	202.3 ± 5.0**fd**
(C) Bean yield per plant[a,b]		
Treatment	gm plant^{-1}	
CF	23.2 ± 1.3	24.9 ± 1.4
NF+O_3	25.7 ± 1.1	19.6 ± 5.0**fd**

[a] All values are reported ± S.E.
[b] Values for cv Forrest superscripted with the letter '**d**' are significantly different at the 95% level from the corresponding value for cv Essex. Values for O_3-treated cv Forrest superscripted with the letter '**f**' are significantly different at the 95% level from values for CF cv Forrest.

removed for area determination. Shoot dry mass data is expressed per plant (9 plants in each of 4 chambers, n = 36 plants).

Since the area of mature upper canopy leaves was measured for each of 9 plants per cultivar per chamber (4 chambers per treatment) then n = 36 leaves per cultivar per treatment. Leaf area is expressed as an individual leaf per plant.

At 144 d PE and after a total of 107 d of low or moderate O_3 exposure, pod fill was complete and beans were ready for harvest. Plants were well past the senescence stage, and the shoot mass was very dry. Ten plants of each cultivar in each chamber were removed carefully in order not to lose any pods or beans. Plants with bean pods still attached were individually bagged and placed in a dry holding area for approximately 1 month to assure total removal of any tissue water. Plants were then removed individually from bags, bean pods were removed and counted; then beans were removed from the pods, counted, and weighed. All yield data is expressed per plant.

Gas exchange measurements

Attached single leaflet net photosynthetic CO_2 assimilation (Pn) rates at 65 d PE (with 40 d of O_3 treatment) and at 74 d PE (with 49 d of O_3 treatment) were measured on plants in the chambers using a LI-COR2, 6200 photosynthesis system (LI-COR, Lincoln, Nebraska) (LI-6200 Primer-Handbook 1987). Photosynthetic CO_2 assimilation (Pn) rate and stomatal conductance (Gs) measurements were made on mature middle leaflets of leaves in the upper canopy of plants within each open-top chamber. Light energy during gas exchange measurements, leaflet chamber CO_2 level (Ce), leaflet internal CO_2 level (Ci), and leaf temperature during measurements are recorded in Table 1.

Leaflet chlorophyll

Leaflet disk samples (6 disks/sample, ≈24.6 cm^2) were collected from control and O_3-treated cultivars at the late vegetative stage (65 d PE and 40 d of O_3 treatment) employing techniques subsequently described. Chlorophyll was extracted from each sample into 100% methanol (100 ml final volume per sample) and quantified spectrophotometrically employing the extinction coefficients derived by MacKinney (1941). Eight samples per cultivar were extracted per treatment and measured.

Leaf disk sampling

Sampling procedures

At the late vegetative stage (65 d PE and 40 d of O_3 treatment) and at the early flowering stage (74 d PE and 49 d of O_3 treatment), leaves of each cultivar were sampled within the open-top chambers. A precalibrated cork borer was used to excise disks (≈4.1 cm^2 per disk) from soybean leaflets; a small sheet of cork backing was positioned behind each leaflet for ease of disk removal. During sampling, 3–6 disks were excised from designated leaves (3 leaflets per leaf), pooled, inserted into 6 × 9 cm manila envelopes, and the envelopes were immediately immersed into liquid N_2. Freezing leaf disk samples in liquid N_2 required approximately 3–6 seconds. The frozen samples were stored at minus 80 °C in an ultra-low temperature freezer until the extraction and quantitation of Chl or ASC and DHA were performed.

Late vegetative stage

Samples were excised twice during the day in the periods 0807–0940 hrs and 0950–1115 hrs. Sampling time for each leaf was recorded and median time points computed (Table 3).

For each time point, 3 plants of each cultivar were selected at random in each of the 8 chambers. One mature leaf (3 leaflets per leaf) from each of the 3 plants per cultivar for a designated time point was selected at random in the top of the canopy. For each leaf at each time point, 2 disks were excised from each leaflet of the leaf, and the resulting 6 disks were pooled into one sample ($\approx$24 cm^2 of leaf tissue). Thus, there were 3 sets of 6 disks per leaf (per plant) collected, and there were 9 samples per cultivar per time point collected for each chamber.

Early flowering stage

Samples were excised three times during the day within the open-top chambers. The first sample period was 0810–0910 hrs; the second sampling period was 1055–1210 hrs and the third sampling period was 1405–1510 hrs.

In this series, an upper canopy mature leaf was selected at random from each of 2 plants per cultivar per chamber per time point. Two samples of 3 disks per sample ($\approx$12 cm^2) were excised from the middle leaflet of each leaf (per plant). Thus, there were 4 samples taken from each cultivar at each time point from each chamber. Sampling time for each leaf was recorded and median time points were computed (Table 4).

In this series (74 d PE), mature leaflet fresh weight per unit area per cultivar per treatment was determined. Twenty disks were selected at random from mature upper canopy leaflets of each cultivar in each of two chambers per treatment. Fresh weights of each set were determined and areas were measured.

ASC and DHA extraction and quantitation

Extraction and quantitation of ASC and DHA from soybean leaf disk samples were carried out following the methods previously described by Luwe et al. (1993) and further modified and thoroughly described by Robinson (1997) and Robinson and Bunce (2000). ASC and DHA were extracted from four samples per cultivar per treatment per time point for plants in the late vegetative stage (65 d PE), and from eight samples per cultivar per treatment per time point for plants in the early flowering stage (74 d PE).

Statistics

Determination of significance between sample means for measured parameters of control and ozonated plants was done employing the StatView II application (Feldman et al. 1987). The Anova-Single Factor Factorial-One Repeated Measure analyses for significant differences between sample means employed the Dunnett *t* test. All mean values are presented $\pm$ S.E.

Results

Vegetative and reproductive yield

At the late vegetative stage (65 d PE and 40 d of O_3 treatment), Essex shoot biomass accumulation per plant was unaffected by elevated O_3 exposure (Table 1A) (n = 36 plants/treatment). In contrast, shoot biomass per plant of cv Forrest plants after 40 d of O_3 treatment was approximately 20% lower than the shoot biomass per plant of control cv Forrest plants in CF chambers (Table 1A).

Cultivar Essex mature leaf areas (3 leaflets/leaf) (n = 36 leaves/treatment) were unaffected by elevated O_3 exposure. Exposure of cv Forrest plants to elevated O_3 resulted in approximately 10% lower mature leaf area than was accrued by control cv Forrest plants (Table 1B).

The influence of O_3 on the bean yield per plant of soybean cultivars Essex and Forrest after 144 days PE and 107 days of elevated O_3 is shown in Table 1C. Cv Essex bean yield (grams) per plant was unaffected by elevated O_3 exposure. Cultivar Forrest bean yield per plant was reduced approximately 20% as a result of exposure to elevated O_3 level.

Leaflet Chl and gaseous exchange parameters

At the late vegetative stage (65 d PE and 40 d of O_3 treatment), Chl per unit area in mature leaflets of control and O_3-treated cv Essex and in mature leaflets of control and O_3-treated cv Forrest was similar. For example, leaflet Chl level in cv Essex control and O_3-treated plants was, respectively, 571.3 ± 19.1 mg m^{-2} and 585.9 ± 9.7 mg m^{-2}. In cv Forrest, leaflet Chl level in control and O_3-treated plants was, respectively, 497.8 ± 6.9 mg m^{-2} and 534.8 ± 17.1 mg m^{-2}. At this growth stage, leaflet Chl levels in cv Forrest plants were slightly, but significantly lower than in leaflets of cv Essex, but this was a varietal difference not associated with the influence of O_3 exposure.

Table 2. Gas exchange measurements for CF and NF+O_3 treatments of cv Essex and cv Forrest in the early flowering stage at 74 d PE with 49 d of O_3 treatment: (1) light energy during measurement, (2) photosynthetic rate during measurement, (3) stomatal conductance during measurement, (4) chamber CO_2 level during measurement, (5) leaflet internal CO_2 during measurement, and (6) leaflet temperature during measurement. Leaflet gas exchange was measured during 1100–1300 hrs (n = 8 measurements per treatment per cultivar)

Cultivar	cv Essex[a]		cv Forrest[a]	
	CF	NF+O_3	CF	NF+O_3
(1) PPF (during Pn measurement)		μmol photons m^{-2} s^{-1}		
	1769 ± 61	1713 ± 133	1796 ± 60	1694 ± 107
(2) Pn rates		μmol CO_2 assimilated m^{-2} s^{-1}		
	17.4 ± 0.9	16.9 ± 0.8	17.1 ± 1.3	17.6 ± 0.8
(3) G_s (during Pn measurement)		mmol H_2O m^{-2} s^{-1}		
	722.3 ± 42.5	727.5 ± 44.9	731.8 ± 89.7	837.7 ± 52.1
(4) Ce (during Pn measurement)		μl CO_2 l^{-1} air		
	327.4 ± 2.2	325.3 ± 2.3	331.9 ± 2.6	323.4 ± 2.4
(5) Ci (during Pn measurement)		μl CO_2 l^{-1} air		
	271.3 ± 2.1	270.8 ± 2.4	275.0 ± 4.5	273.1 ± 1.6
(6) Leaflet temperature (during Pn measurement)		°C		
	34.5 ± 0.3	34.3 ± 0.3	34.2 ± 0.3	34.1 ± 0.3

[a] Absence of the letter '**d**' on a cv Forrest value indicates the corresponding value for cv Essex is not significantly different.

Table 3. Late vegetative stage: Leaflet levels of ASC, DHA and total Vit C (with units as mmol per square meter), and ASC and DHA mole fractions in cv Essex and cv Forrest plants in the CF and NF+O_3 open-top chambers (n = 4 measurements per cultivar per treatment per time point). Samples were taken at random from mature leaflets in the canopy tops. PPF has the units, μmol photons m^{-2} s^{-1}

Treat-ment	Median Time	Ave. PPF	ASC	DHA	ASC+DHA	ASC/total	DHA/total
				mmol m^{-2}		mole fractions	
(A) cv Essex[a,b]							
CF	0838	880	0.92 ± 0.04	0.13 ± 0.01	1.05 ± 0.04	0.88 ± 0.01	0.12 ± 0.01
NF+O_3	0904	963	1.00 ± 0.12	0.19 ± 0.06	1.19 ± 0.06	0.84 ± 0.06	0.16 ± 0.06
CF	1043	545	0.99 ± 0.08	0.13 ± 0.02	1.12 ± 0.06	0.88 ± 0.02	0.12 ± 0.02
NF+O_3	1019	525	1.08 ± 0.07	0.17 ± 0.00	1.26 ± 0.06	0.86 ± 0.01	0.14 ± 0.01
(B) cv Forrest[a,b]							
CF	0847	880	0.78 ± 0.03**d**	0.22 ± 0.02**d**	1.00 ± 0.02	0.78 ± 0.02**d**	0.22 ± 0.02**d**
NF+O_3	0912	963	0.79 ± 0.02**d**	0.29 ± 0.06**d**	1.08 ± 0.05**d**	0.73 ± 0.05**d**	0.27 ± 0.05**d**
CF	1050	545	0.65 ± 0.09**d**	0.28 ± 0.02**d**	0.93 ± 0.07**d**	0.70 ± 0.05**d**	0.30 ± 0.05**d**
NF+O_3	1026	525	0.85 ± 0.00**d**	0.25 ± 0.00**d**	1.10 ± 0.00**d**	0.77 ± 0.00**d**	0.23 ± 0.00**d**

[a] All values are ± S.E.
[b] Values for cv Forrest superscripted with the letter '**d**' are significantly different, at the 95% level, from the corresponding value for cv Essex.

Table 4. Early flowering stage: leaflet levels of ASC, DHA, total Vit C (ASC+DHA) (expressed on a leaf area basis (A, C) and a fresh mass basis (B, D)) and ASC and DHA mole fractions in cv Essex and cv Forrest plants in the CF and NF+O_3 open top chambers. Samples are from mature leaflets in the canopy during three time points (n = 16 measurements per cultivar per treatment per time point). PPFD has the units μmol photons m^{-2} s^{-1}

Treat-ment	Median time	Ave. PPF	ASC	DHA mmol m^{-2} (A, C) μmol g^{-1} fresh mass (B, D)	ASC+DHA	ASC/total mole fractions	DHA/total mole fractions
(A) cv Essex (leaflet area)[a,b]							
CF	0838	488	1.43 ± 0.04	0.24 ± 0.02	1.67 ± 0.05	0.86 ± 0.01	0.14 ± 0.01
NF+O_3	0838	425	1.48 ± 0.04	0.23 ± 0.02	1.71 ± 0.02	0.86 ± 0.01	0.14 ± 0.01
CF	1131	1550	1.63 ± 0.04	0.27 ± 0.02	1.90 ± 0.03	0.86 ± 0.01	0.14 ± 0.01
NF+O_3	1130	1588	1.50 ± 0.05	0.20 ± 0.02	1.70 ± 0.06	0.88 ± 0.01	0.12 ± 0.01
CF	1439	1638	1.63 ± 0.06	0.20 ± 0.01	1.83 ± 0.06	0.89 ± 0.01	0.11 ± 0.01
NF+O_3	1440	1806	1.48 ± 0.06	0.24 ± 0.00	1.72 ± 0.05	0.86 ± 0.02	0.14 ± 0.02
(B) cv Essex (fresh mass)[a,b]							
CF	0838	488	9.30 ± 0.28	1.54 ± 0.11	10.84 ± 0.33	0.86 ± 0.01	0.14 ± 0.01
NF+O_3	0838	425	9.65 ± 0.24	1.50 ± 0.14	11.15 ± 0.15	0.86 ± 0.01	0.14 ± 0.01
CF	1131	1550	10.61 ± 0.28	1.72 ± 0.16	12.33 ± 0.20	0.86 ± 0.01	0.14 ± 0.01
NF+O_3	1130	1588	9.73 ± 0.34	1.32 ± 0.14	11.05 ± 0.41	0.88 ± 0.01	0.12 ± 0.01
CF	1439	1638	10.61 ± 0.36	1.28 ± 0.09	11.89 ± 0.36	0.89 ± 0.01	0.11 ± 0.01
NF+O_3	1440	1806	9.60 ± 0.38	1.61 ± 0.13	11.21 ± 0.29	0.86 ± 0.02	0.14 ± 0.02
(C) cv Forrest (leaflet area)[a,b]							
CF	0841	488	1.16 ± 0.03**d**	0.49 ± 0.03**d**	1.65 ± 0.02	0.70 ± 0.02**d**	0.30 ± 0.02**d**
NF+O_3	0841	425	1.14 ± 0.03**d**	0.54 ± 0.05**d**	1.68 ± 0.03	0.68 ± 0.03**d**	0.32 ± 0.03**d**
CF	1134	1550	0.93 ± 0.09**d**	0.50 ± 0.02**d**	1.43 ± 0.08**d**	0.65 ± 0.04**d**	0.35 ± 0.04**d**
NF+O_3	1132	1588	0.88 ± 0.08**d**	0.59 ± 0.00**d**	1.47 ± 0.03**d**	0.60 ± 0.05**d**	0.40 ± 0.06**d**
CF	1441	1638	1.07 ± 0.10**d**	0.47 ± 0.04**d**	1.54 ± 0.09**d**	0.70 ± 0.04**d**	0.30 ± 0.04**d**
NF+O_3	1443	1806	0.91 ± 0.09**d**	0.57 ± 0.05**d**	1.48 ± 0.06**d**	0.61 ± 0.04**d**	0.39 ± 0.04**d**
(D) cv Forrest (fresh mass)[a,b]							
CF	0841	488	8.12 ± 0.21**d**	3.44 ± 0.17**d**	11.56 ± 0.14**d**	0.70 ± 0.02**d**	0.30 ± 0.02**d**
NF+O_3	0841	425	7.96 ± 0.22**d**	3.83 ± 0.37**d**	11.79 ± 0.17**d**	0.68 ± 0.03**d**	0.32 ± 0.03**d**
CF	1134	1550	6.51 ± 0.63**d**	3.50 ± 0.28**d**	10.01 ± 0.58**d**	0.65 ± 0.04**d**	0.35 ± 0.04**d**
NF+O_3	1132	1588	6.16 ± 0.59**d**	4.13 ± 0.38**d**	10.29 ± 0.23**d**	0.60 ± 0.05**d**	0.40 ± 0.06**d**
CF	1441	1638	7.53 ± 0.70**d**	3.26 ± 0.30**d**	10.79 ± 0.63**d**	0.70 ± 0.04**d**	0.30 ± 0.04**d**
NF+O_3	1443	1806	6.39 ± 0.60**d**	4.01 ± 0.36**d**	10.40 ± 0.39**d**	0.61 ± 0.04**d**	0.39 ± 0.04**d**

[a] All values are ± S.E.
[b] Values for cv Forrest superscripted with the letter '**d**' are significantly different, at the 95% level, from the corresponding value for cv Essex

Pn and G_s measurements of leaflets of late vegetative stage plants (65 d PE and 40 d of O_3 treatment) were carried out on a cloudy day in PPF (330–650 μmol photons m^{-2} s^{-1}) which was rate-limiting with respect to leaflet photosynthetic CO_2 assimilation measured at ambient CO_2 levels (≈340 μl l^{-1} air). Also, in the early flowering stage (74 d PE and 49 d of O_3 treatment), leaflet Pn and G_s measurements were made in high sunlight at PPF (≈1700–1800 μmol photons m^{-2} s^{-1}) which produced maximum Pn rate at ambient CO_2 levels (≈340 μl l^{-1} air). However, regardless of the growth stage or measuring PPF, Pn rates and G_s were not reduced as a result of long-term exposure to elevated O_3 (Table 2 and data not shown).

Mature leaflet ASC and DHA levels and mole fractions

Late vegetative stage

On the late vegetative stage sampling day, the light intensity during the first sampling period (≈0830–0912

hrs) and the second sampling period (≈1019–1050 hrs) was relatively low due to cloud cover (Table 3A, B).

In CF and O_3-treated cv Essex plants, average steady-state levels of ASC, DHA, and total ascorbate, and mole fractions of ASC and DHA in leaflets were similar. By mid-morning, ASC levels increased slightly in cv Essex leaflets, in both control and O_3-treated plants (Table 3A).

During the first morning sampling, average cv Forrest leaflet steady-state levels of ASC, DHA, and total ascorbic acid, and mole fractions of ASC and DHA were also similar in CF and O_3-treated plants. At the mid-morning time point, there was a slight decrease in ASC levels in the leaflets of the CF cv Forrest leaflets. However, in O_3-treated cv Forrest, there was a slight increase of leaflet ASC level in the O_3 treated plants at the mid-morning time point (Table 3B).

The major differences in leaflet ASC, DHA and total ascorbate levels as well as ASC and DHA mole fractions occurred between cultivars, and these differences were seen in the leaflets of both CF plants and O_3-treated plants. While CF and O_3-treated cv Essex leaflets had steady-state ASC levels in the range of 0.92–1.08 μmol m^{-2} (Table 3A), leaflets of CF and O_3-treated cv Forrest had lower ASC levels, e.g. 0.65–0.85 μmol m^{-2} (Table 3B). CF and O_3-treated Essex leaflets displayed DHA levels of 0.13–0.19 μmol m^{-2} (Table 3A), but DHA levels of CF and O_3-treated cv Forrest leaflets were higher, i.e. 0.22–0.29 μmol m^2 (Table 3B).

ASC and DHA levels in leaflets of CF and O_3-treated cv Essex were approximately 86 mole % and 14 mole %, respectively, of the total ascorbic acid (Table 3A). In contrast, ASC and DHA in leaflets of cv Forrest were approximately 74 mole % and 26 mole %, respectively, of the total ascorbic acid (Table 3B).

Early flowering stage

In this stage, leaflet ASC, DHA, and total ascorbate levels are expressed both on an area basis (Table 4A, cv Essex; Table 4C, cv Forrest) and on a fresh mass basis (Table 4B, cv Essex; Table 4D, cv Forrest) in order to demonstrate that trends in the difference in ASC and DHA levels and mole fractions between the two cultivars are the same regardless of the method of quantitative expression.

In leaflets of cv Essex control plants, ASC levels rose 1.14 fold between the morning and midday point and remained at that level into the midafternoon (Table 4A, B). However, in leaflets of O_3-treated cv Essex plants, ASC level remained constant through the day at a level similar to that of the control plant leaflets in the morning point (Table 4A, B). Leaflet DHA levels in control and O_3-treated cv Essex plants varied slightly with no definite trend through the day (Table 4A, B). Total ascorbate (ASC+DHA) levels in cv Essex control plant leaflets displayed a slight increase between the morning and midday point, because of the slight increase in ASC level, and because DHA level did not decrease (Table 4A, B). Steady-state levels of total ascorbate in cv Essex O_3-treated plants displayed no rise or fall because ASC and DHA levels remained relatively unchanged through the entire day (Table 4A, B).

In leaflets of control cv Essex, ASC remained approximately 86 to 89 mole % and DHA was 11 to 14 mole % of the total ascorbic acid (Table 4A, B). In leaflets of O_3-treated cv Essex, ASC was 86 to 88 mole % and DHA was 12 to 14 mole % (Table 4A, B). Ozone treatment did not decrease the ASC:DHA status in cv Essex leaflets.

The data concerning the early flowering stage indicates that throughout the sampling period, ASC levels were always significantly lower, and DHA levels were always significantly higher in leaflets of control and O_3-treated cv Forrest than in leaflets of control and O_3-treated cv Essex (Table 4).

In leaflets of cv Forrest control plants, ASC levels decreased between the morning and the midday point, but then ASC increased between the midday and mid-afternoon point (Table 4C, D). However, ASC levels in the midafternoon sample were slightly but significantly lower than they had been in the first morning sample, e.g. ≈0.6 μmol gm fresh $weight^{-1}$ lower (see Table 4 D). DHA levels in leaflets of cv Forrest control plants remained relatively similar during the sampling period, although they were always more than two times higher than DHA levels in cv Essex control plants (Table 4C, D).

At the same time, ASC levels in leaflets of O_3-treated cv Forrest plants decreased significantly between the morning period and the midday period and continued to remain at that lower level into the midafternoon (Table 4C, D). Not all of the decrease in ASC can be attributed to the oxidation of ASC to DHA. For example, between the morning and midday time point, ASC level declined from 7.96 to 6.16 μmol g^{-1} fresh weight (a decrease of 1.80 μmol) (Table 4D). Concomitantly, DHA level rose from 3.83 to 4.13 μmol g^{-1} fresh weight (an increase of 0.30 μmol) (Table 4D). Of the amount of ASC that disappeared,

only ≈17% of it reappeared as DHA, thus it must be concluded that the other 83% of ASC and/or DHA was metabolized in some pathway other than a redox reaction.

The ASC mole % of the total ascorbate in cv Forrest control plant leaflets shifted from 70% in the morning to 65% at midday and back to 70% by midafternoon (Table 4B, D). Concurrently, DHA mole fractions shifted from 30% in the morning to 35% at midday and back to 30% by midafternoon (Table 4B, D).

In leaflets of O_3-treated cv Forrest, the ASC was 68 mole % of the total ascorbate at the morning point and fell to 60% at midday and remained at that mole fraction through the midafternoon (Table 4B, D). At the same time the DHA mole % increased from 32 to approximately 40% during the same time intervals (Table 4B, D). Thus there was a decline in the redox status which was partially related to the increased oxidation of ASC to DHA (as indicated above). The decrease of leaflet ASC not recovered as DHA in O_3-treated cv Forrest was reflected in the lower level of total ascorbate at the midday and midafternoon points (Table 4B, D).

Discussion

O_3 tolerance and sensitivity in cultivars

Vegetative and reproductive yield of cv Essex plants was unaffected by prolonged exposure to moderate daily elevated O_3 levels (i.e., at 58 nl l^{-1} air), but vegetative and reproductive yield of cv Forrest was significantly decreased by prolonged elevated O_3 levels (Table 1). In many plant species, photosynthetic membranes and enzymes of the pentose phosphate reductive cycle in the mesophyll chloroplasts are damaged by exposure of leaves to very high O_3 levels, e.g. in excess of 100 nl l^{-1} air (Heath 1994, 1996). However, in cv Essex and cv Forrest leaflets, neither CO_2 assimilation nor stomatal conductance per unit leaflet area were diminished by continuous exposure of plants to moderate levels of O_3 (Table 2). Ozone treatment apparently did not directly inhibit and/or damage photosynthetic functions in the mesophyll leaf chloroplasts in the leaflets of either cultivar. However, because mature leaflets of O_3-treated cv Forrest had 10% lower total area than leaflets of control or O_3-treated cv Essex plants (Table 1B), total photosynthate production (e.g. hexose phosphates, glucose, sucrose and ascorbic acid) per leaflet of O_3-treated cv Forrest would have been less over the total growing season. This, in turn, would have contributed to decreased shoot dry mass per plant (Table 1A), and ultimately a decreased bean yield (Table 1C). Thus, over the growing season, exposure of cv Forrest to elevated O_3 level did have an ultimate impact on total foliar photosynthetic productivity, even though active oxygen species apparently did not inhibit carbon assimilation enzymes (Table 2).

Ascorbic acid level and redox status

The apoplast (cell wall) is currently envisioned as the first line of antioxidant defense against O_3-derived active oxygen compounds, e.g. superoxide and hydrogen peroxide (Castillo and Greppin 1988; Polle et al. 1990; Luwe et al. 1993; Polle et al. 1995; Luwe and Heber 1995; Luwe 1996; Ranieri et al. 1996; Lyons et al. 1999; Burkey 1999). Exposure of a variety of plants such as Sedum, spinach, pumpkin, beech, and field beans to elevated O_3 level may cause as much as 10% of the total leaf ASC to diffuse from the leaf mesophyll cell symplasts into the apoplasts where it serves as reductant for the ASC peroxidase catalyzed peroxidation of H_2O_2. During exposure of plants to elevated O_3 levels, peroxidative activity in cell walls causes a considerable decrease in apoplastic ASC and an increase in apoplastic DHA. Whole leaf and symplastic ASC level and ASC–DHA redox status usually decrease only slightly unless the O_3 levels are very high (Castillo and Greppin 1988; Luwe et al. 1993; Luwe and Heber 1995; Luwe 1996; Ranieri et al. 1996; Burkey 1999).

In the current study, we examined ASC and DHA levels in the leaves of field grown soybeans exposed to environmentally relevant, elevated O_3 levels. In control as well as O_3-treated cv Essex plants at the vegetative and flowering stages, leaflet ASC and total ascorbate slightly increased or remained the same throughout the day (Tables 3 and 4); this indicated that there was no net loss of ASC and no net loss of DHA other than from conversion back to ASC. Over many weeks of exposure of cv Essex to elevated O_3, the demands for constant regeneration of ASC in the leaflet mesophyll cell apoplast and symplast apparently were supplied. However, this appeared not to have been the case in the leaflet cells of cv Forrest, because there was consistently a lower ASC mole fraction and higher DHA mole fraction in O_3-treated plants. This suggested that in leaflet mesophyll cells of O_3-treated

cv Forrest, the continuous recycling of DHA–ASC was not as vigorous as it was in the leaflet cells of cv Essex (Tables 3 and 4). Thus the results suggest that the success of cv Essex in maintaining the normal vegetative and reproductive status during O_3 exposure relates partly to its higher daily foliar ASC level and higher ASC–DHA redox status in leaflets of cv Essex (Tables 3 and 4).

In leaflets of cv Essex and cv Forrest (or leaves of any green plant), the net steady-state levels of ASC and DHA at any given time point in the light or dark is the result of simultaneous ASC synthesis, ASC metabolism (degradation), and oxidation-reduction turnover of ASC–DHA to ASC (Loewus 1988; Smirnoff 1996; Noctor and Foyer 1998; Wheeler et al. 1998; Conklin et al. 1999; Davey et al. 1999; Loewus 1999; Pallanca and Smirnoff 1999). It remains a possibility that the higher ASC level and steady-state ASC–DHA redox status in cv Essex leaflets, compared with that of cv Forrest leaflets, reflects more active synthesis of ASC as well as a more rapid turnover of DHA–ASC in leaflet cells of cv Essex and a more rapid degradation of ASC and/or DHA in leaflet cells of cv Forrest. It is also possible that the apoplastic-symplastic recycling of ASC and DHA is slower in the leaf mesophyll cells of cv Forrest than in the leaf cells of cv Essex.

Our comparison of soybean cultivars Essex and Forrest contributes whole leaf evidence that there is an apparent relationship between elevated ASC levels and tolerance to O_3-induced oxidative stress. However, before a direct correlation between the ASC level, ASC redox status and tolerance to O_3 can be established, there must be a thorough characterization of mesophyll cell cytosolic and organelle enzymes associated with ASC synthesis, redox turnover, metabolism (degradation) and mechanisms of ASC and DHA inter- and intracellular transport during periods of O_3-induced oxidative stress. Furthermore, during exposure of plants to elevated O_3, the levels of H_2O_2 in the leaf mesophyll cell apoplast and symplast must be measured concurrently with the levels of ASC and the ASC:DHA status in order to better define a quantitative relationship between ASC and O_3 tolerance.

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